THE "AGGLUTINATION" PHENOMENON WITH SPERMATOZOA OF CHITON TUBERCULATUS

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(*From the Bermuda Biological Station for Research* [1])

Introduction

It has been shown by Crozier (1922) that when sperm, diffusing from a male individual of *Chiton tuberculatus* during the month of May (fully a month before ripe eggs are seen), was taken up between the ctenidia of a female, it issued from the posterior ends of the ctenidial channels principally in the form of "numerous agglutinated masses of active sperms" which persisted in sea-water for at least one half-hour. He found similar "agglutination" when sperm had passed through the ctenidial channels of males, and when it had been added (1) to ovarian extracts from mature eggs in sea-water, or (2) to sea-water into which ripe eggs had been shaken from an ovary and allowed to stand for half an hour. He considered these conditions to indicate that "mere evidence of sperm agglutination (cluster formation) may well have no bearing on the fertilization reaction." This conclusion is in distinct conflict with that formulated by Lillie (1919) on the basis of his observations on *Arbacia punctulata* and *Nereis limbata*.

The observations of Crozier (1922) have been extended during a series of observations made during the summers of 1933 and 1938 at The Bermuda Biological Station for Research.

Observations

Dry spermatozoa [2] from a mature male *Chiton tuberculatus* are homogeneously motile. When such dry sperm is introduced into the mantle cavity either of a male, or of a female which does not shed eggs, in a way such that it is caught up in the ctenidial current and carried

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[2] When a drop of such sperm is brought into contact with a drop of mature eggs on a glass slide, the egg contents, within about one minute, shrink visibly so that a clear area is produced around the egg and between the egg and the chorion. When such eggs are transferred to Syracuse watch crystals containing approximately 10 cc. sea-water, about 98 per cent of them will have cleaved, within approximately one and one-half hours, to form the two-cell stage.

through the gills to be discharged at the posterior end, this discharged sperm, if allowed to collect in the dish, or to rest undisturbed on a glass slide, can be seen to form macroscopically visible white masses in a short time, as Crozier observed. If, on the other hand, the discharged sperm is collected with a clean pipette, transferred to a glass slide, and examined immediately, a very interesting series of changes can be observed.

During the first few seconds, the spermatozoa swim about actively, freely, homogeneously. During the next few seconds, they come together and form small clumps of spermatozoa, *but the point of attachment is the tail*, while the heads remain perfectly free. Sometimes the group may consist of but ten or twelve spermatozoa, in which case the clumps resemble bouquets of flowers, tufts of grass, or even more appropriately, a handful of balloons waving in the breeze. Soon these clumps fuse into larger masses, either in such a way as to form complete spherical masses, as shown in Fig. 1, or much more frequently, to form strands of spermatozoa. These strands, at first, are slender and comparatively short, and are often but slightly branched, but soon they elongate, thicken and branch, as shown in Fig. 2. Soon these strands fuse with others, in a way such that within about three minutes extensive networks appear, as may be seen from Fig. 3. These networks soon become so large that they are macroscopically visible.

Careful observation of the mode of formation of these strands reveals that the process is quite comparable to a braiding of the tails of the spermatozoa. Always at the end of the strand, a tuft of spermatozoa with entirely free heads may be seen. These heads are continually waving back and forth, in and out, moved by the whipping motion of the spermatozoön tail. In a short time, the latitude of the motion becomes restricted, and the restriction progressively increases until finally only the head is free. It can then be seen, still actively waving, from the side of the strand. Often, however, the head is included in the braid.

As the sperm become more and more bound in the strand, other spermatozoa get caught and soon these are inescapably bound while others are caught. This continues until all of the free-swimming spermatozoa are bound, when the terminal tufts then persist for long periods of time, probably permanently. These tufts have been examined with especial care, and in every case, the sperm head has been found to be absolutely distinct and completely separate from its neighbors.

When, however, a drop of dry sperm on a glass slide is brought in contact with a drop of sea-water in a way such that the spermatozoa are carried into the sea-water by the resultant currents, it frequently happens that the sperm suspension is simply swept into the sea-water drop. Unlike the condition with most forms, the sperm mass tends to remain intact, and though it generally exhibits a slight increase in homogeneous motility, it shows no slightest sign of the clumping phenomenon. Occasionally, however, under such conditions, tufts of spermatozoa may form along the contacting edge, as may be seen in Fig. 4.

On the other hand, when the two drops are fused in a way such that the spermatozoa are distributed widely and rapidly into the sea-water, *all* stages that were observed with the sperm that had passed through the ctenidial channels could be seen repeated under such conditions. The phenomenon was first noted when the sperm drop was brought into contact, in the usual way, with a drop of sea-water to which a little ether had been added. The markedly reduced surface tension produced violent currents which served rapidly to carry the sperm to all parts of the drop. The ether evaporated rapidly, and by so doing probably produced still more currents, but did not appear to affect the motility of the sperm in any way. Later it was possible to repeat exactly the same series of changes with non-etherized, normal sea-water by the proper regulation of the relative sizes of the drops. When the two drops are fused with the aid of a clean glass needle in a way such that the sperm suspension is spread widely throughout the drop of sea-water, the typical strands, as shown in Fig. 2, form immediately and everywhere, and these soon anastomose to form the extensive net-works shown in Fig. 3. In these nets, the sperm heads frequently project into the interstices of the net and there continue to wave actively for a long period of time. Sometimes the network formation may be so extensive that distinct membrane-like structures are produced, which readily curl up and may readily be caused to wave in a manner typical of any such membrane, if the slide be moved or shaken gently under the microscope.

When a drop of dry sperm is introduced suddenly into 8 or 10 cc. of sea-water in a small beaker, the sperm mass may be seen to drop to the bottom of the beaker in the form of a much-folded membrane, resembling in every respect a piece of silk allowed to fall lightly on a table. Microscopic examination of this mass shows that it is composed of a still intact mass of homogeneously distributed spermatozoa. Though this membrane of spermatozoa is very delicate, it is possible to lift it, *as a membrane*, with a fine glass needle, and to fold it back upon itself, or to roll it into a markedly more compact mass. Tufts, in due time, appear along the edges of the mass, and circular clumps may

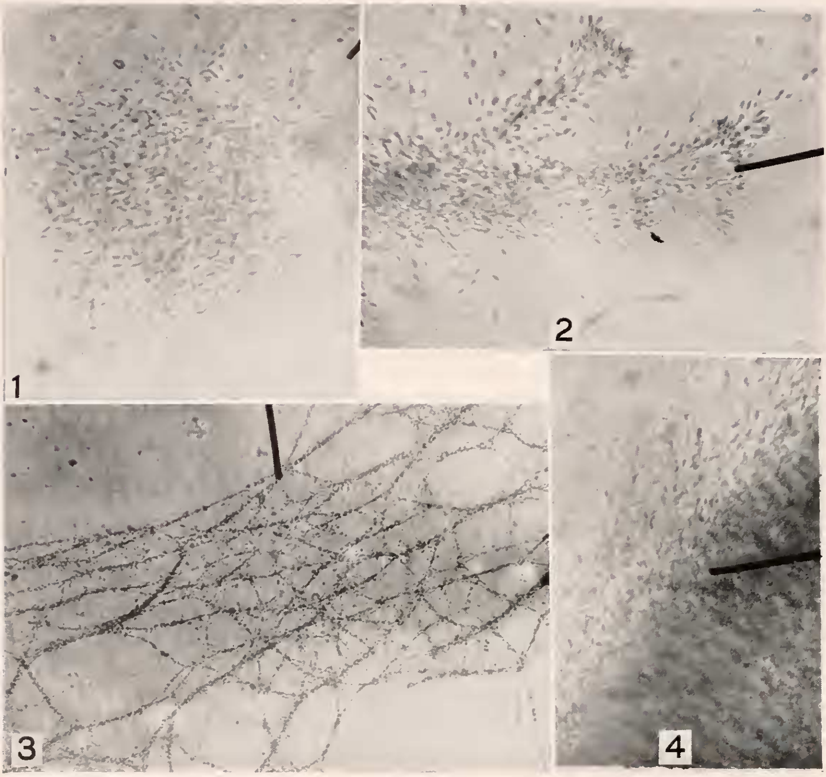

PLATE I

Fig. 1. The structure of the spherical masses that are formed by the spermatozoa of *Chiton tuberculatus* after dilution with sea-water, or after passage through the ctenidial chambers of a male or immature female. Sometimes the masses formed are more compact, but the structure, with the tails bound and the heads free, is the same in all cases. × 440.

Fig. 2. The elongating, thickened, branching strands that form when the spermatozoa of *Chiton tuberculatus* is passed through the ctenidial chambers of a male or immature female, or is diluted with sea-water. × 440.

Fig. 3. The nature of the network that forms by the fusion of the strands produced by the spermatozoa of *Chiton tuberculatus* when diluted with sea-water, or passed through the ctenidial chambers of a male or immature female. × 100.

Fig. 4. Photomicrograph to show the retention of mass integrity, with occasional formation of tufts along the contacting edge, when a drop of dry spermatozoa of *Chiton tuberculatus* is gently brought into contact with a drop of sea-water. × 440.

appear separated from, but near the margins of, the membrane. Rarely, strands may also appear. When, however, the original sperm mass becomes spread very thin, widespread formation of the network usually results.

If, however, some of the sperm be separated from the main mass, either by means of a glass needle, or by mechanical agitation, typical strands and networks are immediately formed. Any mechanical disturbance of the drop of dry sperm in order to obtain a uniform, homogeneous, diluted sperm suspension results only in a complete transformation of the sperm mass into strands, and a microscopic examination of sperm suspensions of one drop of dry sperm to 5, 10, 15, 25, 50, 100, and 150 cc. sea-water have all shown the presence of such strands, and the complete absence, in any case, of completely free swimming spermatozoa. These strands slowly settle to the bottom of the container, the rate depending upon their size, where they form into networks, and, if the concentration be sufficient, into more or less homogeneous membranes. These membranes can again be broken up into strands, and the process can be repeated several times. The strands in sea-water persist indefinitely, or as long as the spermatozoön tail is intact. In the more dilute suspensions, however, the strands remain quite uniformly distributed in the suspension for some time.

On the other hand, when extracts from the ovaries of ripe or spent females, obtained by cutting the ovary into small pieces and washing it thoroughly through cheese-cloth with about 25 cc. of sea-water, are used instead of sea-water, *no clumping in any form occurs.* Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop is reached. At no time do tufts, strands, or networks appear, and, furthermore, if spermatozoa be drawn from the mass by means of a glass needle, they simply disperse and soon merge imperceptibly with the other spermatozoa as the main mass advances upon and closes in about them.

Dilution of the ovarian extracts appears to lessen their effectiveness. A dilution of one drop of extract to ten drops of sea-water is often quite as effective as the full strength extract. A 1 : 25 dilution, however, prevents the formation of tufts and other similar structures, but does not remove the restriction to a free movement of the sperm through the drop of ovarian extract; while a 1 : 50 dilution allows the formation of tufts, clumps, strands, and networks just as though it were pure sea-water. These figures, however, are of relative value only, since there is a wide variation between the extracts from the different ovaries, as seen in the fact that one of those tested gave a perfectly typical clumping reaction with a 1 : 10 dilution. Clumps of spermatozoa formed by dilution of dry sperm with sea-water can be caused to disperse by the addition of ovarian extract.

When egg-sea-water, made by allowing the eggs of one female to stand in 25 cc. sea-water for half an hour, was used, exactly similar effects were obtained to those obtained with the ovarian extracts. Of course, dilution of this egg-sea-water reduced the effect, just as was the case with the ovarian extract. When, too, a drop of dry sperm was brought in contact with a drop of eggs and watched under the microscope, the sperm could be seen to move freely across the open spaces, and to gather about the eggs, but there was no sign whatsoever of a clumping reaction. These latter observations are in accord with, and to an extent provide an explanation of, the observations of Crozier (1922) that "during natural fecundation, however, no sperm-balls are formed. The thick, glutinous stream of spermatozoa passes under the girdle of the female, is somewhat diluted with sea-water by the tractive current, and emerges posteriorly in company with numerous large greenish eggs, about which, under the microscope, it can be seen that many sperms are gathered. But no real 'cluster formation' takes place."

Other substances that prevent clumping include the body juices of a mature male, body juices of a mature female, as was also noted by Crozier (1922), sublethal solutions of saponin in sea-water,[3] similar solutions of sodium taurocholate in sea-water, saturated and somewhat diluted solutions of trypsin in sea-water, and possibly by other substances. On the other hand, no prevention of clumping was obtained with acetone, ether, methyl alcohol, ethyl alcohol, adrenaline chloride (1 : 1000, 1 : 2000, or 1 : 5000), or carbon disulphide.

Discussion

It is obvious that in *Chiton tuberculatus*, the clumping reaction is different in every way from the agglutination reaction which has been described by Lillie (1913) for *Arbacia punctulata*, by Loeb (1914) for *Strongylocentrotus purpuratus* and *S. franciscanus*, by Just (1919) for *Echinarachnius parma*, and later (1929) for *Paracentrotus lividus* and *Echinus microtuberculatus*, by Carter (1932) for *Echinus esculentus*,

[3] The sources of the substances used for these tests were as follows:

Saponin: Eimer and Amend, New York, "A-61 Purified."
Sodium taurocholate: Eimer and Amend, New York, "A-61 Purified."
Trypsin: Eimer and Amend, New York, "A-61" "Pure."
Acetone: U. S. P., J. T. Baker Chemical Co., Lot No. 92237.
Adrenaline Chloride: Parke, Davis and Co., Detroit, Mich., U. S. A.
Ether, methyl alcohol, ethyl alcohol, and carbon disulphide: Usual laboratory supplies.

by Lillie (1913) for *Nereis limbata*, by Just (1915) for *Platynereis megalops*, and by other workers with other forms. With the agglutination reaction in these cases, the agglutination is between the heads of the spermatozoa, while the tails are apparently unaffected; the agglutination reaction is spontaneously reversible, cannot be repeated, and generally is produced only by substances secreted by eggs of the same species. With the clumping reaction in *Chiton tuberculatus*, on the other hand, the agglutination is between the tails while the heads are apparently utterly free and unaffected; the clumps, when once formed, persist indefinitely unless dispersed by means of the addition of certain substances, or by mechanical means. In the latter case, the clumps will reappear with an almost endless number of repetitions. The clumping forms as a perfectly natural and normal result of dilution with ordinary sea-water, and is, in addition, a phenomenon which can be prevented by means of body juices of the same form, egg and ovarian secretions and extracts, and certain lytic substances, such as saponin and sodium taurocholate.

With *Chiton tuberculatus*, it is unlikely that the clumping reaction, first described by Crozier, and described in detail herein, has any *direct* relation to the fertilization reaction as such. Instead, it is probably a mechanism by means of which masses of dry sperm may be transferred, in an intact manner, from the male to the mature female. This mass of dry sperm, thus transferred in an essentially intact condition, comes in contact with substances in the ctenidial channels of the female which destroy the substance which causes the tails to stick together and thus form clumps. The spermatozoa thus become freed from each other and are then able, by their own individual and utterly independent movements, to activate the all-environing eggs. The reason for transferring the spermatozoa to the female in an *intact* mass, however, might possibly reside in the need to preserve substances which might be essential for the actual fertilization reaction, and which might be rapidly lost from the spermatozoön in less concentrated suspensions.

The fact that these clumps can be dispersed by means of the proteolytic enzyme, trypsin, and by the lytic substances saponin and sodium taurocholate indicates that the clumping reaction in this form rests, fundamentally, upon the presence, on the outside surface of the spermatozoön tails at the time they are liberated from the testes, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by the above-mentioned substances. Since lytic substances, as suggested by Ponder (1930), might act by the destruction of the structure of proteins, a process which is also hastened by trypsin, it is possible that the substance on the tails of the spermatozoa of dry sperm suspensions might be a sticky protein of some sort. On the other hand, Fieser (1937) has suggested that the hemolytic effect, of saponin at least, might be produced by a combination with cholesterol or lecithin of the cell membrane in a way such as to render the membrane permeable to hemoglobin, and evidence in partial support of this suggestion has been provided by Ransom (1901) in that he has shown with certainty that a combination of saponin and cholesterol is possible and that treatment of a saponin solution with cholesterol destroys its hemolytic activity. Popa (1927) has obtained evidence that the tails of the spermatozoa of *Arbacia punctulata* and *Nereis limbata* are enveloped by a large amount of lipoid substance.

Summary

1. When dry sperm of a mature male *Chiton tuberculatus* is introduced into the mantle cavity of a male or immature female and the discharged sperm is collected and examined immediately with a microscope, the spermatozoa will be seen to come together and form small clumps. *The point of attachment of these clumps is the tail*, while the heads remain perfectly free.

2. These clumps fuse readily to form either large spherical masses, or strands, which, in turn, soon fuse with other strands to form extensive networks. Such structures also form, readily and extensively, when the two drops are fused with the aid of a glass needle in a way such that the sperm suspension is distributed widely throughout the drop of sea water.

3. On the other hand, when extracts from the ovaries of ripe or spent females, egg-sea-water, body juices of a mature male, or of a mature female, or sublethal solutions of the lytic substances, saponin or sodium taurocholate, are used, *no clumping in any form occurs.* Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop of diluting fluid is reached.

4. These observations indicate that, in *Chiton tuberculatus*, the clumping reaction rests fundamentally upon the presence, on the outside surface of the spermatozoön tails, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by certain substances.

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